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Estimation of Nitrifying Bacterial Activities by Measuring Oxygen Uptake in the Presence of the Metabolic Inhibitors Allylthiourea and Azide

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The effects of two metabolic inhibitors on an enriched nitrifying biomass during incubation for short periods of time were investigated by determining respirometric measurements. Allylthiourea (86 μM) and azide (24 μM) were shown to be strong, selective inhibitors of ammonia and nitrite oxidation, respectively. Consequently, a differential respirometry method for estimating nitrifying and heterotrophic bacterial activities within a mixed biomass is proposed.

Nitrogen removal is partially achieved by nitrification, which is performed by two groups of gram-negative, obligately aerobic bacteria. Ammonia oxidizers and nitrite oxidizers transform ammonia to nitrite and nitrite to nitrate, respectively (7, 19).

Several authors have estimated kinetic parameters of nitrification by obtaining respirometric measurements (oxygen depletion due to substrate consumption) for pure cultures (6, 9), activated sludges (16, 17), or biofilms (11). For mixed cultures containing heterotrophs and ammonia and nitrite oxidizers, selective inhibitors that allow separation of the different activities are needed (2, 15, 16). As suggested previously (2), the inhibition should be instantaneous and complete for the targeted population and should not affect other populations.

Allylthiourea selectively inhibits ammonia oxidation at concentrations between 8 and 80 μM (6, 8, 15, 16), probably by chelating the copper of the ammonia monooxygenase active site (2). Chlorate has been used to stop nitrite oxidation in soils, sediments, and activated sludge systems (3, 10, 16, 17). However, doubts concerning the slow and nonspecific action of chlorate limit its usefulness in discriminatory respiratory assays with mixed cultures (3, 10), as confirmed by us previously (data not shown). Azide (N_3^-) has been shown to be a selective bacteriostatic agent that is active against gram-negative bacteria (12) and to be an inhibitor of ammonia and nitrite oxidation in activated sludge (18). Azide also inhibits the nitrate reductase of denitrifiers, which contain a molybdenum cofactor like the nitrite oxidoreductase of *Nitrobacter* spp. (5, 7, 13). Since there are similarities between denitrifying nitrate reductases and nitrite oxidoreductases (7), we tried to use azide as a selective inhibitor of nitrite oxidation.

The nitrifying biomass used in this study was enriched and developed from an activated sludge (from Morainvilliers, France) by repeated lab subculturing in mineral medium (14) over several months. This biomass contained nitrite and ammonia oxidizers and, presumably, heterotrophs. The acetate-dependent oxygen uptake rate was very low (less than 3 mg of $O_2 \cdot h^{-1} \cdot g$ of $protein^{-1}$) compared to the nitrite- and ammonia-dependent respiration rates (which were between 39 and 352 mg of $O_2 \cdot h^{-1} \cdot g$ of $protein^{-1}$) (Table 1), suggesting that the culture was highly enriched for autotrophic nitrifying microorganisms. It is also possible that the acetate-dependent oxygen uptake was due to nitrite oxidizers which are capable of growing mixotrophically (19). The nitrifying biomass also exhibited oxygen uptake activity in the absence of exogenous substrate, which is defined as endogenous respiration (4, 11). Separation of endogenous respiration, acetate-dependent respiration, nitrite-dependent respiration, and ammonia-dependent respiration necessitated estimation of the concentrations at which inhibitors had selective, complete, and instantaneous effects. Consequently, a protocol which allowed differentiation between bacterial activities in a mixed culture containing nitrifiers is described below.

Experiments based on oxygen uptake measurements were developed to ensure that azide (99% pure; Merck, Darmstadt, Germany) and allylthiourea (97% pure; Rhône-Poulenc Ltd., Manchester, United Kingdom) completely and instantaneously inhibited nitrite and ammonia oxidizers, respectively, without affecting other activities. Oxygen uptake measurements were obtained with a batch type respirometer (model 5300 biological oxygen monitor; Yellow Springs Instruments, Yellow Springs, Ohio) at 20°C and pH 7.6. Washed biomass was added to two incubation chambers filled with 10 ml of oxygen-saturated mineral medium (14).

As expected, allylthiourea selectively inhibited ammonia oxidizers at a concentration of 86 μM without affecting other activities (Table 1), and 80% inhibition of ammonia oxidation was observed at a concentration of 1 μM (Fig. 1); these results are similar to results obtained previously with *Nitrosomonas europaea* (6, 10). The complete inhibition observed after 10 min of exposure to 10 μM allylthiourea was not instantaneous (Fig. 1). Instantaneous, complete inhibition was observed at an allylthiourea concentration of 86 μM (Fig. 1).

At a concentration of 24 μM, azide did not affect the endogenous, ammonia-dependent, and acetate-dependent respiration rates but did instantaneously and completely inhibit nitrite oxidation (Table 1 and Fig. 2). In addition, the inhibition was independent of the nitrite concentration and was reversible after azide was removed by biomass washing (data not shown). This is the first report in which azide is described as an inhibitor of nitrite oxidation in vivo (50% inhibition at a concentration of 0.3 μM) (Fig. 2). In vitro, azide completely inhibited nitrite oxidation in cell extracts of *Nitrobacter agilis* at

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TABLE 1. Influence of inhibitors on the different types of respiration

Type of respiration	Specific oxygen uptake rate in the presence of no inhibitor (control) (mg of $O_2 \cdot h^{-1} \cdot g$ of protein^{-1})[a]	Specific oxygen uptake rate (% of control value) in the presence of[b]:	
		Allylthiourea (86 μM)	Azide (24 μM)
Endogenous (no substrate)	12–17 (11)[c]	95 ± 5 (4)	98 ± 5 (4)
Acetate (10 mg of C/liter)	1–3 (4)	94 (1)	101 ± 10 (2)
Nitrite (10 mg of N/liter)	39–120 (23)	99 ± 2 (3)	1 ± 1 (10)
Ammonia (10 mg of N/liter)	137–352 (14)	0 ± 1 (10)	98 ± 0 (2)

[a] Calculated by determining the difference between oxygen uptake rates before and after substrate was added.
[b] Calculated as described in the legends to Fig. 1 and 2.
[c] The numbers in parentheses are numbers of experiments.

a concentration of 100 μM, although lower concentrations were not studied by the authors who performed this study (1). Previous results for the purified nitrate reductase of a denitrifying bacterium showed similar inhibition (13). As the nitrite oxidoreductase system is able to act as a nitrate reductase in the absence of oxygen (i.e., nitrate is transformed to nitrite) and both enzymatic systems contain a molybdenum cofactor (6, 8, 13), we assume that azide could act by complexation with the molybdenum atoms of the nitrite oxidoreductase.

Since the bacteriostatic effect of azide on gram-negative bacteria is well known (12), this compound is also likely to inhibit ammonia oxidizers. Azide was less effective in inhibiting ammonia oxidation than in inhibiting nitrite oxidation (Table 1 and Fig. 2); activity was inhibited 75% at concentrations of 2,800 and 0.8 μM with ammonia and nitrite oxidizers, respectively (Fig. 2). Thus, we assume that azide is a selective inhibitor of nitrite oxidation in a mixed bacterial population when the concentration is low (less than 24 μM).

Since allylthiourea (86 μM) and azide (24 μM) are selective, instantaneous, and effective inhibitors of ammonia and nitrite oxidizers respectively, a respirometric test that differentiates between endogenous respiration (by heterotrophs and/or nitrifiers) and exogenous heterotrophic and nitrifier respiration (by ammonia and nitrite oxidizers) within a mixed biomass may be described (Fig. 3). The addition of a mixture of energetic substrates (ammonia, nitrite, and acetate) to a substrate-free biomass suspension leads to intense oxygen uptake, which is subsequently and selectively inhibited by allylthiourea (86 μM) and azide (24 μM) (Fig. 3).

Substrate concentrations and pH effects, which are two environmental factors likely to affect the usefulness of this procedure, were studied in separate experiments. An acceptable compromise which resulted in the optimal response for the whole system (i.e., maximal activity and no toxicity phenomena at high nitrogenous substrate concentrations) was found to be 10 to 20 mg of N (nitrite and ammonia) per liter and pH 7.6 (data not shown).

This method is very simple and could be extended to characterization and control of other nitrifier-containing environmental samples, such as activated sludges (Fig. 3) and biofilms, provided that the sensitivity to inhibitors is similar. This

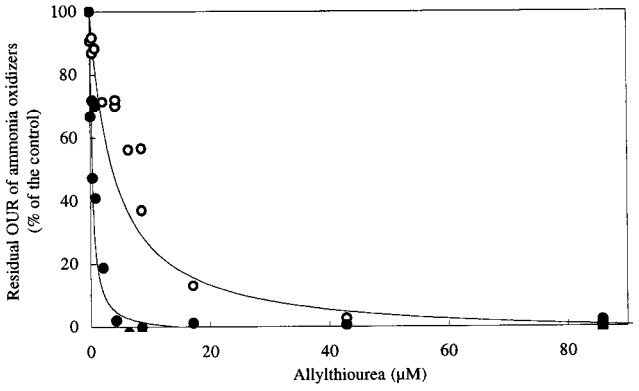

FIG. 1. Residual oxygen uptake rate (OUR) of ammonia oxidizers after 0.5 (○) or 10 (●) min of exposure to allylthiourea (0 to 86 μM). A washed biomass suspension incubated with no substrate but with azide 24 μM (nitrite oxidizers were therefore inactive [Table 1]) was supplemented with ammonia (10 mg of NH_4^+ N · liter^{-1}) and, after 5 min, with allylthiourea (0 to 86 μM). Values were obtained by comparing the oxygen uptake rates of ammonia oxidizers (from which endogenous oxygen was removed) before and after a 0.5- or 10-min exposure to allylthiourea. The endogenous oxygen uptake rate was not affected by 86 μM allylthiourea (Table 1).

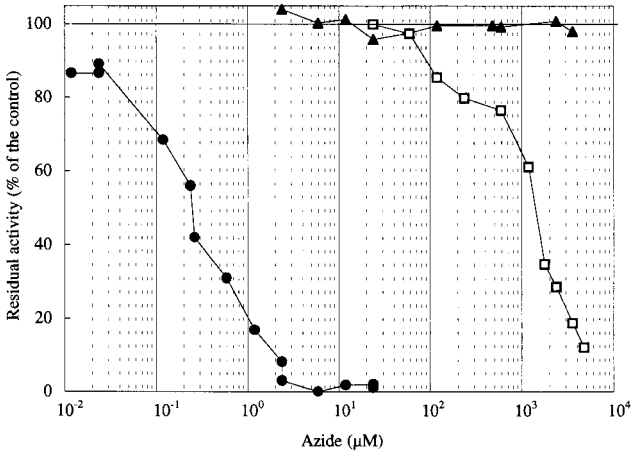

FIG. 2. Effect of azide on endogenous (▲), nitrite-dependent (●), and ammonia-dependent (□) oxygen uptake rates. For endogenous respiration, the oxygen uptake rates of a washed biomass incubated with no substrate (endogenous oxygen uptake rate) before and after addition of azide (0 to 4,800 μM) were compared, which yielded the percentage of residual activity for endogenous respiration. For nitrite oxidizers, in the presence of 10 mg of NO_2^- N · liter^{-1} the oxygen uptake rates (from which endogenous rates were removed) before and after addition of azide (0 to 24 μM) were compared, which yielded the percentage of residual activity for nitrite-dependent respiration. For ammonia oxidizers, a washed biomass supplemented with 10 mg of NH_4^+ N · liter^{-1} and 10 mg of NO_2^- N · liter^{-1} was subsequentially inhibited by azide (24 to 4,800 μM) and, after 5 min, by allylthiourea (86 μM). The difference between the oxygen uptake rates before and after allylthiourea was added yielded the oxygen uptake rate for ammonia oxidizers in the presence of azide. This value was compared to the value obtained in a control experiment, which was similar except that the inhibitors were introduced in an inverse order (i.e., ammonia oxidizer activity in the absence of azide), which yielded the percentage of residual activity for ammonia oxidizers in the presence of azide. The endogenous oxygen uptake rate was not affected by 4,800 μM azide.

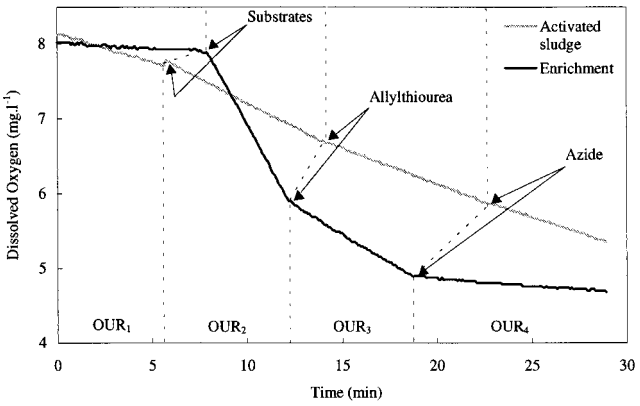

FIG. 3. Typical profiles for oxygen uptake by an enriched nitrifying biomass (106 mg of protein · liter^{-1}) and an activated sludge sample (Morainvilliers, France; 225 mg of protein · liter^{-1}). Samples (pH 7.6, 20°C) were supplemented with a mixture of substrates (10 mg of NH_4^+ N · liter^{-1}, 10 mg of NO_2^- N · liter^{-1}, and 10 mg of acetate C · liter^{-1}) and subsequently inhibited by allylthiourea (86 μM) and azide (24 μM). From this respirogram, endogenous respiration and ammonia, nitrite, and acetate oxidation activities may be calculated by determining the following oxygen uptake rates (OUR): OUR_1, $OUR_2 - OUR_3$, $OUR_3 - OUR_4$, and $OUR_4 - OUR_1$, respectively.

method could also be used to study the toxicity of a given compound for the different fractions of a mixed culture.

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